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**14. ABSTRACT**

ATF3 is a newly-identified p53 regulator, whose expression is frequently down-regulated in prostate cancers. The overall goal of this project is to elucidate the role of ATF3 in prostate carcinogenesis. Towards this aim, we are in a process of determining whether ATF3-deficient mice are prone to prostate carcinogenesis (Specific Aim #1) as well as whether ATF3 deficiency promotes survival of prostate epithelia cells (Specific Aim #2). Our major task in this report period (~6 months)is to establish a colony of ATF3 knockout (KO) mice in our animal facility and allow it to expand for subsequent studies. We have successfully completed this task, and obtained ~30 KO mice – 10 of them will be housed until moribund to determine whether aged mice grow prostate lesions (Task #1) while others will be bred with WT mice and ARR2Pbi-Cre/Pten(-/-) mice for carcinogenesis studies. In addition, we have developed several ATF3-knockdown LNCaP cell lines using a retroviral-based shRNA technology. These cells will be used to determine the effects of ATF3 on the survival of prostate cancer cells (Task #4).

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## INTRODUCTION

This study will determine the role of activating transcription factor 3 (ATF3) in prostate carcinogenesis. ATF3 is a novel stabilizer of p53, which promotes the tumor suppression functions of the latter protein. Given that ATF3 can be induced by a large variety of oxidative stresses, our hypothesis is that ATF3 activates p53 in response to aging-related increase of oxidative stress thereby preventing the occurrence as well as progression of prostate cancer. This study aims at testing this hypothesis by using both genetically-engineered mouse models and *in vitro* cell culture systems. It was initiated on January 8, 2007 at the University of Texas M. D. Anderson Cancer Center, but adjourned on February. 23, 2007 due to the relocation of the PI to Albany Medical College. The study was resumed on August 15, 2007 after the grant has been successfully transferred to Albany Medical College. This report covers the research in the first year that is ended on Jan 7, 2008, and spans about 6 months.

## BODY

According to the approved Statement of Work, the main tasks of the first 6 months (the period covered by this report) are (a) to breed and genotype ATF3 KO mice (Task #1, a) and (b) to knock down ATF3 expression in LNCaP cells (Task #4, a). We have successfully completed these tasks, and the results are described in details as follows.

### *Breed and genotype ATF3-/- mice (Task #1, a)*

One of the Specific Aims of this study is to utilize genetically-engineered mice to determine whether ATF3 deficiency promotes prostate carcinogenesis. ATF3 knockout (KO) mice (C57/BL6 background) have already been developed by Dr. Tsonwin Hai at the Ohio State Univerisity (OSU) (1). Upon the approval of ICAUC of both DoD and Albany Medical College (AMC), 2 female and 3 male KO mice were delivered from OSU to the AMC Animal Research Facility, and housed in a quarantine room (for infection clearance) for 2 months before transferred to a SPF room in the Facility. Two breeding pairs were set up, which generated 4 male and 6 female offspring. The offspring were used to set up 3 breeding trios, yielding ~30 mice (half male and half female) by the end of the report period. The mice grew normally, although their weights were less than that of WT pups. Because of this, young KO mice were waned at 30 days of age instead of 21 days to ensure survival.

To confirm that the offspring contain KO allele, we developed a PCR-based genotyping method based on a previous publication (1). As shown in Fig 1A, the KO mice were developed by replacing Exon 2 of the *atf3* gene with a neomycin gene (*Neo*). We synthesized 3 primers so that primers 186 (5'-AGAGCTTCAGCAATGGTTTGC-3') and 187 (5'-TGAAGAAGGTAAACACACCGTG-3') can amplify a region spanning 329bp in the WT allele while primers 186 and 188 (5'-ATCAGCAGCCTCTGTTCCAC-3') amplify a 236bp fragment in the KO allele (Fig 1A). Therefore, a PCR with a mixture of 3 primers at a ratio 2:1:1 (186:187:188) can distinguish a KO mouse (yielding a 236 bp product) from a WT mouse (a 329 bp product) or a heterozygous mouse (both 236-bp and 329-bp products).

For genotyping, we prepared genomic DNA from 30-day old mice. Thus, 0.5cm of mouse tails were cut with a pair of sharp scissors, and digested with 100 μg/ml proteinase K in a buffer containing 100 mM Tris(pH8.5), 0.5mM EDTA, 2% SDS and 200 mM NaCl at 55°C overnight. After centrifuge, genomic DNAs in supernatants were precipitated with equal volume of isopropanol and harvested by centrifuge. The DNAs were washed with 75% ethanol, air dried, and dissolved in 50 μl TE buffer. For PCR, 2 μl DNA was added in a 20 μl reaction containing the primer mixture, and subjected to a 35-cycle PCR including a denature step at 94°C for 30 sec, an anneal step at 57°C for 30 sec and an extension step at 72°C for 30 sec. As expected, genomic DNA from the offspring yielded a single PCR product with a size less than 250 bp – less than that from WT mice, demonstrating that the offspring contain homozygous KO allele (Fig 1B). We randomly chose 10 of male offspring, and group them for aging-related prostate carcinogenesis studies (Task #1). Female offspring are planned to cross with WT C57/BL6 mice that will be purchased from Taconic Farms to generate heterozygous mice. Further breeding with these heterozygous mice will yield littermates with both WT and KO genotypes, and they will be grouped for sex-hormone-induced carcinogenesis (Task #2) and castration-induced prostate epithelial cell death studies (Task #6). The female KO mice will also be used to breed with $Pten^{-/-}$ mice and ARR2Pbi-cre mice for Pten-deficiency-induced carcinogenesis studies (Task #3).

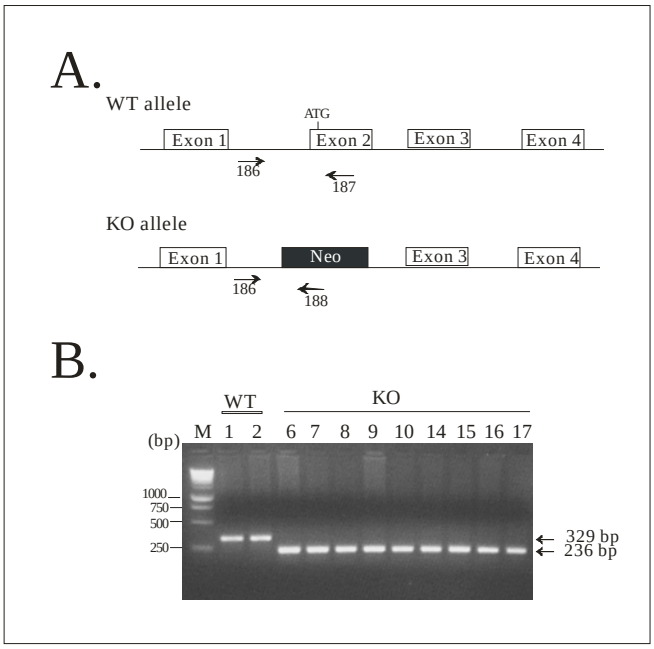


**Fig 1. Genotyping of ATF3 KO mice.** (**A**) Diagram showing ATF3 WT and KO alleles. The positions of 3 primers used for genotyping are indicated by arrows. (**B**) Genomic DNA were prepared from WT and KO mouse tails and subjected to PCR as described in the text. PCR products were resolved in 2% agarose gel. M: Promega 1 Kb DNA marker.

***Development of LNCaP clones in which ATF3 expression was knocked down (Task #4, a)***

We have completed a task to develop LNCaP clone in which ATF3 expression was knocked down by RNA interference (Task #4, a). These cells are required for the investigation of the role of ATF3 in regulating prostate cancer growth (Task #4). To complete this task, we first identified two ATF3 mRNA regions that could be efficiently targeted by siRNA based on our previous study (2). We thus purchased four individual siRNA instead of a siRNA pool (2) from Dharmacon, transfected them into A549 cells and then determined their effects on ATF3 expression by Western blotting. As shown in Fig 2, siRNA #1 and #3 could reduce both the basal and camptothecin (CPT)-induced ATF3 expression by more than 70% in the cells. The

sequences for regions targeted by siRNA#1 and siRNA #3 are 5'-GCAAAGTGCCGAAACAAGA-3' and 5'-GAGAAACCTCTTTATCCAA-3', respectively.

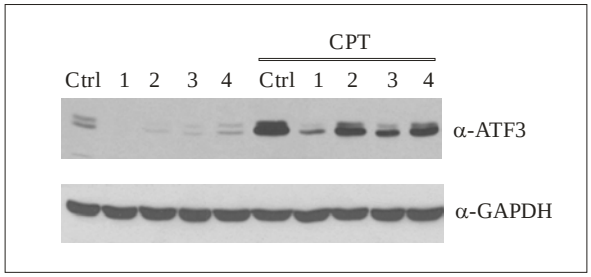


**Fig 2. Identification of two ATF3 mRNA regions for RNA interference.** A549 cells were transfected with four synthesized siRNA or a scamble control (Ctrl) for 3 days followed by treated with 5 μM CPT or ethanol for 4 h. Cell lysates were subjected to Western blotting for ATF3 expression.

Accordingly, we synthesized two 60nt complementary oligoes, which contain a hairpin sequence, a sense and an anti-sense target sequence, as well as Bgl II and Hind III restrictive sites, for each region (3). After annealing, the DNA was ligated into a Bgl II/Hind III-linearized pSuperior vector. The ligated DNA was then amplified in E. coli, and sequenced to verify that no mutation existed. We then transfected these plasmids into Ampli-293 cells to allow them be packed into retroviral particles, while the supernatants containing viral particles were used to infect LNCaP cells in the presence of 4 μg/ml polybrene. We incubated the infected cells in a medium containing 2 μg/ml puromycin for about 3 weeks until resistant clones grew up. After expansion, the cells were lysed and subjected to Western blotting to determine ATF3 protein level.

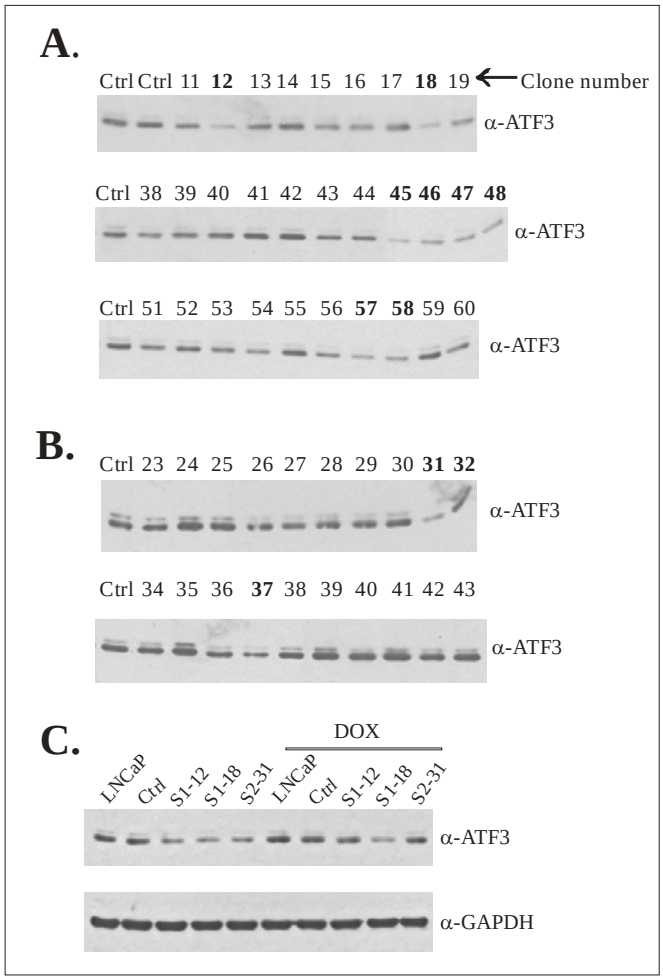


**Fig 3. Establishment of LNCaP stable clones with reduced ATF3 expression.** pSuperior vectors containing shRNA expression cassettes targeting at Region #1 (**A**) and #3 (**B**) were packed into retroviral particles for infection of LNCaP cells. After selected with 2 μg/ml puromycin, resistant clones were expanded and lysed for Western blotting for ATF3 expression. The clones with reduced ATF3 protein levels were marked by number in bold. (**C**) These clones were also tested for basal or DOX-induced ATF3 levels after prolonged culture (2-3 weeks). Only two clones (S1-18 and S2-31) remained low ATF3 levels. The cells were treated with 0.2μg/ml DOX for 4 h.

We have obtained a total of 60 resistant clones that could express short-spin RNA targeting at region #1. Of them, 6 clones were found with reduced ATF3 expression level (Fig 3A, clones #12, 18, 45, 46, 47, 48, 57, 58). Similarly, we obtained 3 such clones after infection of LNCaP cells with a vector containing the target region #2 (Fig 3B, clones #31, 32, 37). Most of these clones, however, appeared to be unstable. After cultured for one to two weeks, the ATF3 protein levels were resumed in these clones. Only clone S1-18 and clone S2-31 remained with low ATF3 level, but the extents of reduction were lower than expected (Fig 3C). The increase of ATF3 levels in these clones might be caused by graduate silencing of the shRNA expression cassettes, an event which frequently occurs after transgenes are integrated into non-permissive genomic locations that are marked by "closed" chromatin structure (4), or adaptive selection if ATF3 is critical for the growth of LNCaP cells. We will utilize these 2 clones to determine the effects of ATF3 down-regulation – which is frequent in prostate cancers – on cell growth (Task #4).

## KEY RESEARCH ACCOMPLISHMENTS

1. A colony of ATF3 KO mice has been successfully established in our animal facility. This is the prerequisite for the completion of the Specific Aim #1, which is to determine the role of ATF3 deficiency in prostate carcinogenesis using genetically-engineered animals. Moreover, 10 male KO mice have been randomly chosen and are housing for aging-related carcinogenesis studies (Task #1).
2. Two stable clones in which ATF3 expression is down-regulated through RNA interference have been developed. This is the first step toward the accomplishment of Task 4 as described in Statement of Work.

## REPORTABLE OUTCOMES

1. ATF3 KO mice have been successfully bred.
2. Two LNCaP cell clones with reduced ATF3 expression have been developed.

## CONCLUSION

During the period covered by this report, we have successfully bred and genotyped ATF3 KO mice while two ATF3-down-knocked LNCaP clones have been developed. These accomplishments constitute the grounds for further studying effects of ATF3 deficiency on prostate carcinogenesis and progression, and are the first steps toward the attainment of the overall goal of this project. The ATF3 KO mice will cross with WT mice and Pten-deficient mice to determine the role of ATF3 gene in preventing prostate carcinogenesis. Ultimately, the knowledge gained from this study will enrich our understanding of the etiology of prostate cancer, resulting in effective therapies fighting against prostate cancer.

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## APPENDICES

None